APPENDIX

The instant application suggests that growing high-density 3-D stromal culture requires a continuous flow of growth media through 3-D carriers settled within the plug-flow bioreactor. The rationale being that a flow system allows the passage of oxygen and nutrients to the cells and removal of waste materials from the cells through an active transfer rather than by diffusion.

The present invention anticipates that stromal 3-D cultures grown in a static system, similarly to the system described by Naughton and co-workers cannot reach a sufficient density to support the survival and expansion of hematopoietic stem cells and progenitor cells, which is the essence of the present invention.

The results presented hereinbelow demonstrate the superior ability of the 3-D stromal cultures, which are grown in the presence of continuous medium flow to support growth of hemoatopoietic stem cells (HSCs) and progenitor cells as compared to the static conditions described by Naughton and co-workers.

Experimental Procedures

Stromal cells –Primary human marrow stromal (6-weeks old) cells were grown in 3-D culture in a plug flow bioreactor for 40 days

Hematopoietic cell-growth in the presence and absence of flow –CD34+ cells were seeded into the bioreactor described in the instant application, which contained a confluent 3-D culture of 40-day old primary human marrow stroma. As a control, CD34+ Cells were also seeded onto confluent static 3-D ("carrier + stroma" bar of Figures 1a-c) or 2-D cultures ("2D stroma" bar of Figures 1a-c) of primary human stroma cells or on 3-D structures without stroma cells ("carrier" bar of Figures 1a-c). The cells were seeded in LTC medium in the absence of cytokines [LTC medium: DMEM (GIBCO BRL), 12.5% heat-inactivated FCS (Beit Ha'Emek, Israel), 12.5% horse serum (HS) (Beit Ha'Emek, Israel), Pen-Strep-Nystatin mixture (Beit Ha'Emek, Israel),10^{-4} M L-glutamine (Beit Ha'Emek, Israel),10^{-4} M mercaptoethanol (Merck), 10^{-6} M hydrocortisone sodium succinate (Sigma)].

7 days following seeding, the cultures were trypsinized and hematopoietic stem cells and progenitors were analyzed by FACS, using the surface markers CD34, CD38 and CXCR4 [Anti-CD34 - fluorescein isothiocyanate (FITC) B&D, NJ, USA, Anti-CD34 - B&D, NJ, USA , Anti-CD38 - phycoerythrin (PE), Coulter, Florida,

USA, Anti-CD45 - fluorescein isothiocyanate (FITC)B&D, NJ, USA, Anti-CXCR4-fluorescein isothiocyanate (FITC) B&D, NJ, USA]. .

Brief description of the figure

FIGs. 1a-c are histograms showing the growth of CD34+ (Figure 1a), CD34+CD38- (Figure 1b) and CD34+CD38-CXCR4+ (Figure 1c) cells under the following test conditions: **Bioreactor total** - Hematopoietic cells growing in the bioreactor (3-D co-cultures + 3-D SCM); **3-D CM** – non-adherent hematopoietic cells collected from the medium circulating in the bioreactor; **Bioreactor carriers**- hematopoietic cells collected from the carriers in the bioreactor; **2-D CM** – non-adherent hematopoietic cells growing in 2-D static co-cultures; **2-D stroma** – adherent hematopoietic stem cells growing in static 2-D co-cultures; **Carrier** - hematopoietic cells growing on carriers in static cultures (without stroma); and **Carrier + stroma** - hematopoietic cells growing on 3-D static co-cultures taken from the bioreactor (similar to the system described by Naughton and co-workers.

CD34+38-CXCR4+/CD34+38-/CD34+ cells input were 115,500, 531,300, and 3,077,000 respectively.

Results are presented as the number of CD34+38-CXCR4+/ CD34+38-/ CD34+ cells taken from three independent samples in two separate experiments.

Results

6-week old primary human marrow stromal cells were grown within the bioreactor for 40 days. Prior to seeding hematopoietic stem cells onto the stromal cells cultures, the primary human stromal cells 3-D culture originating from bone marrow was validated not including hematopoietic cells, which might influence the results of the experiment. In order to verify it, 3-D cultures of stromal cells were sterilely removed from the bioreactor and examined for the presence of hematopoietic precursors, essentially, presence of the CD34 membrane marker (data not shown).

In the next stage, confluent 3-D cultures of hematopoietic-free stroma cells were co-incubated with hematopoietic stem cells (HSCs) and the growth of HSCs under flow conditions was compared to the growth of HSCs under static cultures, by cell counting and FACS analysis of the membrane markers CD34, CD38 and CXCR4.

Noteworthy is that, following 4-24 hours most of the HSCs which were seeded on the 3-D stroma cells cultures were found to be embedded within the stroma.

As shown in Figures 1a-c, the 3-D structure could support the growth of CD34 + 38- and CD34 + 38- CXCR4+ cells better than the suspension cultures and the stroma cells 2-D cultures. Nevertheless, following 7 days of incubation only 30% of the initially seeded CD34+ cells remained (Figure 1a), and the number of CD34 + 38- (Figure 1b) and CD34 + 38- CXCR4+ (Figure 1c) decreased to less than 5% of the seeded cell number (i.e., input).

The static 3-D primary human stroma cells cultures that were previously removed from the biorcactor and kept in petry dishes (carrier + stroma) supported the CD34+ better than the control groups containing 3-D structures (without stroma) or 2-D stroma cultures. Noteworthy is that the static 3-D cultures (similar to the system described by Naughton and co-workers, see "carrier + stroma" bar) could not support the maintenance of hemoatopoietic stem cells and progenitor cells and the remaining CD34 + 38- and CD34 + 38- CXCR4+ cells in the static cultures were only 21% and 10% (respectively) of the initial cell number, which indicates the inability of the static cultures to support HSCs.

In sharp contrast, the plug flow bioreactor, which contained the 3-D primary human stroma cells cultures, supported the growth of CD34+ cells significantly better (i.e., 3 fold, see "bioreactor total" bar vs. "carrier + stroma" bar) than the static 3-D stroma cells cultures. The 3-D culture contained within the bioreactor supported the different hematopoietic precursors more than 10 fold better than control groups containing the 2-D cultures or the 3-D carrier structure (without stroma cells). Unlike the static 3-D cultures ("carrier +stroma" bar), which did not support the CD34+ 38- cells, the bioreactor containing 3-D stroma cells cultures supported the expansion of these cells. The CD34+ 38- cells were found within the stroma cells 3-D cultures (45%) and within the growth media (55%).

Similar results were found in connection with the ability of the bioreactor system to support CD34+ 38-CXCR4+ cells (Figure 1c). While 3-D stroma cells static cultures could hardly support the maintenance of less than 10% of the seeded CD34+ 38- CXCR4+ cells, the plug-flow bioreactor system was able to support 2-fold expansion of these cells. 60 % of these HSCs were found within the circulating growth media and 40% were found within the 3-D stroma cells cultures.

Altogether, these results prove the need for a flow system bioreactor to support the 3-D stroma cells cultures to thereby allow HSCs expansion.

Fig. 1a

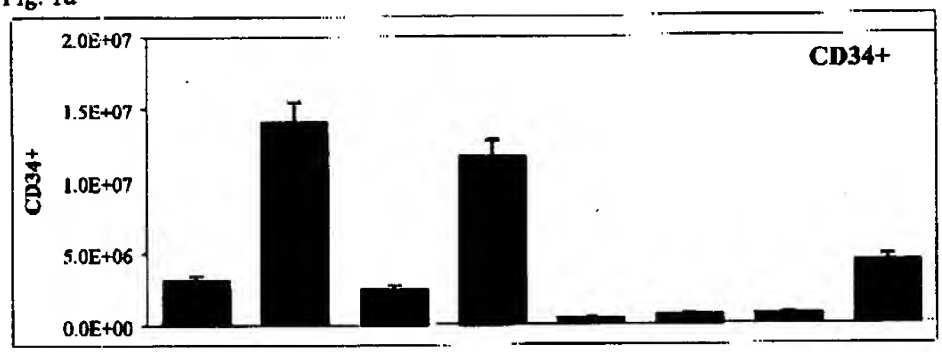

Fig. 1b

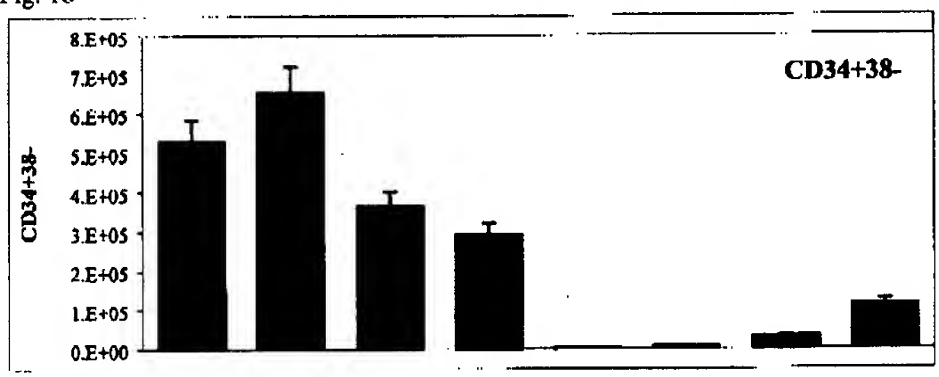

Fig. 1c